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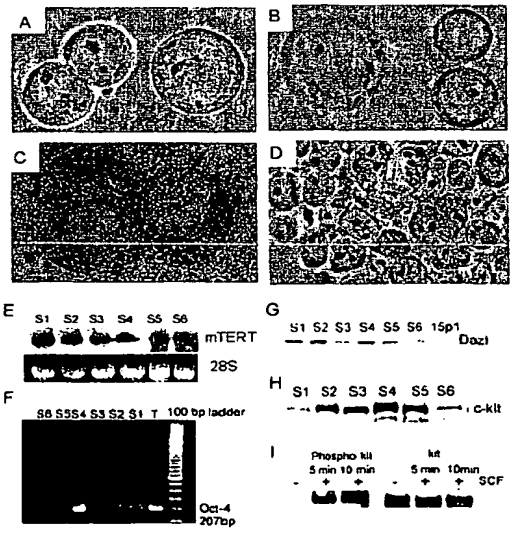
(54) Title: SPERMATOGONIAL CELL LINE




(57) Abstract: The invention provides methods for in vitro generation of a telomerase-immortalized spermatogonial cell line. The invention further provides for the generation of spermatocytes and spermatids from immortalized spermatogonial cells by differentiation in the presence of stem cell factor. This differentiation can occur in the absence of supportive cells. Immortalized spermatogonial cell lines provide powerful tools for elucidating molecular mechanisms of spermatogenesis and for screening and developing compounds which affect spermatogenisis. Furthermore, through genomic modification and transplantation techniques, this male germ cell line can be used to generate transgenic animals and in germ cell gene therapy.

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# SPERMATOGONIAL CELL LINE

## BACKGROUND OF THE INVENTION

### Field of the Invention

**[0001]** The invention relates to the making and using of a spermatogonial cell line.

### Description of the Related Art

**[0002]** Spermatogonia originate from primordial germ cells (PGCs), which are derived from the epiblast by 7.5 days post coitum (dpc) and migrate through the dorsal mesentery and enter the developing fetal gonad, the genital ridge, between 10.5 and 12.5 dpc (1,2). Once they arrive in the genital ridge, the PGCs are enclosed by somatic Sertoli cells and become prospermatogonia or gonocytes (3). The gonocytes proliferate for a few days and then arrest in the G0/G1 phase until birth. Within a few days after birth the gonocytes resume proliferation to initiate spermatogenesis. And by day 6 postpartum (PP), these cells migrate to the basement membrane of the seminiferous tubules and become undifferentiated type A spermatogonia, the male germ-line stem cells (4). Type A spermatogonia either renew themselves to maintain the pool of stem cells or undergo differentiation to produce spermatozoa (5). The male germ-line stem cell expresses high levels of telomerase activity but during germ cell differentiation, telomerase activity is progressively lost (6). Furthermore, telomerase deficiency in mice leads to a depletion of male germ cells (7).

**[0003]** There have been efforts in the past to provide methods for germ cell gene therapy and the generation of transgenic species. For example, Brinster and Zimmerman have shown spermatogenesis following male germ-cell transplantation, providing methods to reimplant spermatogonial cells into sterilized recipients (15). In another approach, Redhead et al. discloses the injection of genetic material directly into the testicle of an animal or the isolation, transfection, and reimplantation of testicular cells (24). The spermatogonial stem cell transplantation technique has the potential to become a powerful tool to create transgenic mice.

Recently, transgenic mice were produced by retroviral transduction of male germ-line stem cells (31). However, the efficiency and the definition of integration of exogenous genes into the genome remain to be improved. It is very difficult to transfer genes into primary cultures of spermatogonia and generally not possible to select the transfected primary cells with drugs *in vitro*. It was previously reported that meiotic cells and spermatids were generated from a male germ cell line established by co-transfecting the simian virus 40 large tumor antigen gene and a mutant of p53 (28). However, a subsequent report by the same group could not confirm the original work (29).

**[0004]** There has been a long felt need in the art for improved methods for the study of spermatogenesis, improved methods for the creation of transgenic animals, and for germline gene therapy (5). For these and other reasons, there is a need in the art for methods of generating immortalized spermatogonial cell lines.

## SUMMARY OF THE INVENTION

**[0005]** The present invention provides methods for the creation of a spermatogonial cell line comprising obtaining undifferentiated spermatogonial cells, preferably purified undifferentiated cells; transfecting the cells with a nucleic acid comprising a sequence encoding for a catalytically active telomerase or fragment thereof; causing the telomerase to be over-expressed in the cells, and culturing the cells. In preferred embodiments, the undifferentiated spermatogonial cells are type A spermtogonial cells obtained from an immature animal. In a more preferred embodiment, the catalytically active telomerase subunit is the telomerase reverse transcriptase (TERT) subunit. In addition to the elements for controlling overexpression of the catalytically active telomerase or fragment thereof, the nucleic acid can also comprise a selectable marker gene such as a gene providing antibiotic resistance. Thus, the method can also comprise selecting for cells that have been transfected, such a selection process can be used to obtain a stably transfected immortalized cell line.

[0006] In an example of a preferred embodiment, undifferentiated type A spermtogonial cells are obtained from an immature male mouse by the STAPUT method at about 6 days post coitum and a nucleotide sequence encoding the mTERT catalytic fragment of telomerase is introduced into the undifferentiated cells by a retroviral vector which also allows for selection of stably transfected cells using G418 in cell culture. The cells of the cell lines created according to the inventive methods can be cultured by standard methods in DMEM/F12, optionally supplemented with fetal bovine serum (FBS). In preferred embodiments, the cells of a cell line made by the methods disclosed herein can maintain the morphology of freshly isolated cells for greater than two months in cell culture, or for greater than 1 year in cell culture in a more preferred embodiment.

[0007] In another aspect of the invention, cells of the cell lines generated by the foregoing methods can be induced to undergo spermatogenesis by exposing the cultured cells of the cell line to stem cell factor (SCF) (for example, about 100 ng/ml). In a preferred embodiment of the invention, about 50% or more of the cells can be induced to form haploid spermatids, for example, in about three weeks.

[0008] In another aspect of the invention, the invention provides a method of investigating the effect of a genetic modification on spermatogenesis comprising obtaining a cell line according to the invention, generating a genetic modification by introduction of an exogenous nucleotide sequence, and observing the effect of the change. Optionally, the method can further comprise using SCF to stimulate spermatogenesis in some or all of the cells.

[0009] In another aspect of the invention, the invention provides a method of investigating the effect of a compound of interest on spermatogenesis comprising obtaining a cell line according to the invention, exposing the cells to a compound of interest, and observing the effect of the compound on the cells. Optionally, the method can further comprise using SCF to stimulate spermatogenesis in some or all of the cells. The effect of the compound of interest can be observed before and/or after SCF induction of spermatogenesis, for example, by morphology, by

chromosomal changes, by measuring the level of expression of a specific marker gene, or by measuring the resulting trascriptional profile.

**[0010]** In an another aspect of the invention, the invention provides a method for the creation of transgenic animals comprising obtaining a cell line according to the invention, generating a genetic modification by introduction of an exogenous nucleotide sequence, optionally selecting for stable incorporation of the transgene in the chromosomal DNA, inducing spermatogenesis using stem cell factor (SCF), and using the resulting spermatids or sperm to fertilize an egg *in vivo* or *in vitro*.

**[0011]** The transgene can be introduced using any appropriate technique recognized in the art. In a preferred technique, the cultured cells of the cell line are exposed to naked DNA encoding the transgene or a genetic modification that can be incorporated by homologous recombination. In an alternative embodiment of the method, spermatogonial cells of the invention that have been transfected with an exogenous nucleotide sequence can be transplanted into the testes of an animal sterilized by chemical or radiological sterilization. The recipient animal is then bred to produce transgenic offspring.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0012]** The objects and advantages of the invention will become apparent from the following detailed description of the preferred embodiments thereof in connection with the accompanying drawings, in which:

**[0013]** FIG. 1. Shows characterization of mouse type A spermatogonial cell line. (A) After two months culture, the immortalized cells possessed the typical morphology of type A spermatogonia such as spherical nuclei and organelles in a perinuclear location. (B) Morphology of freshly isolated type A spermatogonia (control). (C) The immortalized cells were positive for c-kit shown by immunocytochemistry. (D) After subcloning and culture for one year, the immortalized cells still maintained the morphology of type A spermatogonia. (E) Northern blot (25 $\mu$g total RNA for each lane) showing mTERT expression in

S1-S6 cell lines. (F) Expression of Oct-4 in immortalized cells shown by RT-PCR as a 207 bp product; lane T, control testis from new-born pups. (G) DAZL was expressed in all 6 cell clones shown by Western blot; the 15p-1 Sertoli cell line is a negative control. (H) c-kit was expressed in all 6 cell clones shown by Western blot. (I) After stimulation with mSCF (100 ng/ml) for 5 and 10 min, c-kit immunoprecipitates were probed for phosphotyrosine and reprobed for c-kit.

**[0014]** FIG. 2. Shows characterization of differentiated cells. (A) Synaptonemal complexes in S4 cells after 7 days culture with 100 ng/ml of mSCF shown by immunofluorescence with SCP3 antibody. (B) Crossovers shown by Giemsa staining in induced S4 cells (arrowheads). (C, D) Immunochemistry and Western blot showing LDH-C4 expression in induced S4 cells. (E) Acr3-EGFP stably transfected S4 cells (control). (F) mSCF induced Acr3-EGFP stably transfected S4 cells. The population of GFP positive cells included large, round cells that are pachytene spermatocytes and round spermatids, which were smaller and possessed a tiny green dot (arrowheads), the proacrosmal granule. (G to I) Round spermatids stained with DAPI (blue; darker). Different stages of the formation of acrosome shown by Acr3-EGFP (green; lighter).

**[0015]** FIG 3. (A) Differential interference contrast showing a granule in a spermatid (arrowhead). (B) Western blotting showing SP-10 expression was induced by SCF; T is a control from adult testis. (C) RT-PCR for protamine-2 expression; T, adult testis; 1, non-RT control for S4 cells after 3 weeks induction; 2, 1 week induction; 3, 2 weeks induction; 4, 3 weeks induction.

**[0016]** FIG 4. Shows flow cytometry analysis. (A) Adult mice testes were digested and the cell mixture was fixed with 70% ethanol and stained with PI. DNA content was measured to show cell ploidy. (B) S4 cells before induction; the percentage of the 4N cell population is 5.4%. (C) After 1 week induction with mSCF, 39% of the cells were 4N. (D) Haploid cells were produced at 58.5% of the total cell population after 3 weeks of SCF induction.

**[0017]** FIG. 5 Shows spermatids made created by exposing mouse spermatogonial cells to stem cell factor at 100 ng/ml. Most of the larger cells are

spermatocytes at various stages of developement. The smaller cells to the left of the pipette tip are haploid round spermatids that have been sequestered and are ready for injection into mature eggs.

**[0018]** FIG. 6 Shows the injection of a mouse spermatid into an egg. Panel A shows the oocyte prior to injection. The zona pellucida surrounding the oocyte and the polar body at the right side of the oocyte are visible. Panel B shows the zona pellucida penetrated and the track of the pipette inside the cell. A spermatid can bee seen in the pipette. Panel C illustrates that the pipette is inserted deep into the oocyte. Panel d shows a single spermatid injected into the oocyte as far from the penetration site as possible. A second spermatid can be seen in the pipette, ready for the next oocyte injection.

**[0019]** FIG. 7 shows development of a mouse egg fertilized by a spermatid made from an immortalized spermatogonial cell line. Panel A shows the egg at 6 hours after spermatid injection. Panel B shows a 2-cell embryo. Panel C shows an 4-cell embryo. Panel D shows an 8-cell embryo.

**[0020]** FIG. 8 shows five mouse embryos at the morula stage.

## DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

**[0021]** There has been evidence that ectopic expression of the telomerase catalytic component, telomerase reverse transcriptase (TERT), in certain cell types can extend their lifespan and even immortalize them (8, 9). Although telomerase is highly expressed in most transformed cells, there is clear evidence that over-expression of TERT can immortalize cells without transformation (8,9). TERT has been used to establish a number of cell lines (30).

**[0022]** We have discovered that spermatogonia can be maintained as a cell line for an extended period of time in an undifferentiated state by over-expression of TERT *in vitro*. Thus the invention provides a method of generating a spermatogonial cell line comprising the steps of obtaining a culture of undifferentiated spermatogonial cells, transfecting the cells with DNA encoding TERT, and culturing the cells. Cells can be cultured in any appropriate media and

conditions recognized in the art for culturing animal cell lines. A preferred media is DMEM/F12 supplemented with fetal bovine serum (10). In a preferred embodiment of the invention, spermatogonial cells are obtained from immature testes which provides for a higher proportion of undifferentiated cells.

**[0023]** Methods for isolating spermatogonial cells are described for example by Bellvé and colleagues (11). In a more preferred embodiment of the invention, purified undifferentiated type A spermatogonial cells are obtained using the STAPUT method as described by Dym and colleagues (10).

**[0024]** As an example, isolation of type A spermatogonial cells from immature animals can be accomplished by a method comprising the following steps. Testes are excised and decapsulated. Seminiferous epithelial cells are then dispersed and separated by methods such as described by Bellvé and colleagues (11) with minor modifications as described below. Briefly, the decapsulated testes are suspended in DMEM/F12 medium containing collagenase (e.g. about 1.5 mg/ml) and DNAse (e.g. about 1 $\mu$g/ml) and incubated at 34° C for 15 min in a shaking water bath operated at 100 cycles/min. After two washes in DMEM/F12 medium, seminiferous cord fragments mostly devoid of interstitial cells are incubated in DMEM/F12 medium containing collagenase (1.5 mg/ml), hyaluronidase (e.g. about 1.5 mg/ml), trypsin (e.g. about 0.5 mg/ml), and DNAse (e.g. about 1 $\mu$g/ml) for about 20-30 min using the conditions described above. The dispersed cells are washed twice with medium and filtered through 80 $\mu$M and 40 $\mu$M nylon mesh, successively. The cells of the dissociated seminiferous epithelium are separated by sedimentation velocity at unit gravity at about 4 °C, using about a 2-4% BSA gradient in DMEM/F12 medium. The fastest sedimenting cells are primarily the type A spermatogonial cells preferred for use in generating spermatogonial cell lines, thus the desired cells can be purified from the other cells present. The cells are bottom-loaded into a SP-120 chamber in a volume of about 30 ml and a bovine serum albumin (BSA) gradient was generated using about 275 ml of about 2% and about 4% BSA. The cells are allowed to sediment for a standard period of about 2.5 hr, and then about 35 fractions of about 15 ml volume are collected at

approximately 90 second intervals. The cells in each fraction are examined under a phase contrast microscope and fractions containing cells of similar size and morphology are pooled and spun down by low speed centrifugation, and then resuspended in DMEM/F12 medium. All chemicals are available commercially, for example, from Sigma Chemical Co. (St Louis, MO).

**[0025]** Any technique recognized in the art for the expression of exogenous DNA in mammalian cells can be used to introduce DNA encoding TERT. However, primary spermatogonia are difficult to transfect. Thus, a preferred technique for the introduction of TERT encoding DNA is by a retrovirus system. Of course, the vector can also comprise any suitable elements recognized in the art that provide for the expression of the TERT sequence, for example the native promoter or promoters capable of driving overexpression of the TERT encoding sequence in animal cells. Each species of animal has a native TERT sequence; one of skill in the art knows how to isolate and clone these sequences into appropriate vector systems. It is not necessary to utilize the TERT sequence native to the animal from which the spermatogonial cells are extracted. However, it may be advantageous to use the TERT sequence of the animal.

**[0026]** For example, a mouse TERT (mTERT) retrovirus construct can be generated by inserting mTERT cDNA into the EcoR I site of the retrovirus vector pLXSN. A 3441 bp EcoR I/EcoR I fragment carrying 3395 bp of full-length murine telomerase reverse transcriptase (mTERT) cDNA was obtained from pGRN188 and subcloned into EcoR I site in a retrovirus vector pLXSN (Clontech Laboratories, Inc.). The orientation for expressing sense mTERT by 5' LTR was checked by digestion with Xho I. Expression of Neor was driven by the SV40 early promoter. Viruses were harvested from supernatants of transfected Phoenix packaging cells. The isolated spermatogonial cells can be infected with a TERT encoding retrovirus vector by following a standard protocol (12).

**[0027]** To select stably transfected cells, a selective marker gene such as *neo* can be provided on the vector. The spermatogonial cell line can be cultured in the presence of a corresponding selective agent (e.g., G418 for the *neo* gene), for a

period of time to allow the cells to expand as they become immortalized. For example, the cells can be cultured for 1 week or more, preferably the cells are cultured for about two months prior to subcloning. Immunocytochemistry can be used to detect the expression of a biochemical marker for spermatogonia such as c-kit.

**[0028]** Clones can be obtained by subcloning techniques recognized in the art. In a preferred embodiment, cells of the cell lines of the invention continue to display a morphology similar to freshly isolated spermatogonia and maintain the expression of telomerase as shown by Northern blot. For example, the cells can maintain the morphology of freshly isolated spermatogonia and the expression of telomerase for two months to a year or longer.

**[0029]** To further confirm the germ cell characteristics of the cells of the invention, the cells can be checked for a germ cell specific RNA-binding protein, DAZL (13), and/or Oct-4, a germ cell specific transcriptional factor (14) by RT-PCR and Western blot. Oct-4 is expressed in totipotent embryonic cells. After gastrulation, Oct-4 expression becomes restricted to primordial germ cells (PGCs). For example, in male mice, the expression of Oct-4 is maintained until the beginning of spermatogenesis and is confined to the type A spermatogonia (16). Thus, where the immortalized cells are derived from type A spermatogonia the derivation can be confirmed.

**[0030]** Differentiation can be induced by exposing cultured spermatogonial cells stem cell factor (SCF) (for example, about 100 ng/ml). In vivo, type A spermatogonia go through mitoses forming many interconnected cells that enter meiosis (17). SCF and its receptor c-kit play a critical role in regulating this initial stage of spermatogenesis and trigger proliferation/differentiation of type A spermatogonia (18). A point mutation in c-kit, which impairs SCF-mediated activation of phosphatidylinositol 3-kinase, leads to complete male sterility due to lack of differentiating spermatogonia (19, 20). Each animal species has its own native SCF, preferably the SCF of the species from which the cells are derived is used. For example, a mouse spermatogonial cell line can be treated with mSCF to

induce spermatogenesis in vitro. Response to SCF can be confirmed by observing tyrosine phosphorylation of c-kit. The response can be observed within a few minutes of mSCF treatment. A substantial proportion of cells can reach the haploid (1N) number of chromosomes characteristic after two to three weeks of SCF induction. In the process of induced differentiation, it is believed that the telomerase expression decreases as is observed in the normal course of differentiation. Use of a feeder layer of cells can help to promote differentiation and sperm development including the generation of mature sperm.

**[0031]** The method can be applied to any vertebrate animal including human. It is preferred that the primary undifferentiated spermatogonial cells be obtained from immature animals because of the much higher proportion of undifferentiated cells, such as type-A spermatogonial cells. However, with sufficient attention to the purity of the undifferentiated primary cells, it will be possible to apply the method to cells obtained from a more mature animal.

**[0032]** Cell lines generated by the method of the invention can be used to examine spermatogenesis in vitro at the molecular level. The cells of the cell lines are easier to transfect than primary cells so that any method of in vitro gene transfer recognized in the art can be used to introduce exogenous DNA sequences into the cells. For example a transfection promoting agent, for example Lipofectamine® or the like, may be used or DNA may be introduced by transfection of naked DNA. In a preferred embodiment, one or more native genes or their control elements can be modified by homologous recombination. The effect of the molecular changes on spermatogenesis can be observed following SCF induction, for example, by morphology, by chromosomal changes, by measuring the level of expression of a specific marker gene, or by measuring the resulting trascriptional profile. Accordingly, the invention provides a method of investigating the effect of a genetic modification on spermatogenesis comprising obtaining a cell line according to the invention, generating a genetic modification by introduction of an exogenous nucleotide sequence, and observing the effect of the change. Optionally, the method can further comprise using SCF to stimulate spermatogenesis in some or all

of the cells. Though the use of this method, the cell lines of the invention will be a useful model system to analyze the mechanisms of infertility caused by genetic factors.

**[0033]** The cell lines of the invention are also useful as a model system to analyze the mechanisms of infertility caused by chemical toxicity. Further, the cell lines of the invention can be used to screen drugs and compounds for desired properties such as the capacity to stimulate or inhibit spermatogenesis. Accordingly, the invention provides a method of investigating the effect of a compound of interest on spermatogenesis comprising obtaining a cell line according to the invention, exposing the cells to a compound of interest, and observing the effect of the compound on the cells. Optionally, the method can further comprise using SCF to stimulate spermatogenesis in some or all of the cells. The effect of the compound of interest can be observed before and/or after SCF induction of spermatogenesis, for example, by morphology, by chromosomal changes, by measuring the level of expression of a specific marker gene, or by measuring the resulting transcriptional profile.

**[0034]** Use of the spermatogonial cell line can resolve current challenges with primary cultures of isolated spermatogonia and greatly increase the success and the efficiency in generating transgenic mice. Moreover, use of the spermatogonial cell line can resolve challenges associated with the current practice of using embryonic stem cells to make transgenic animals. The common practice of the prior art utilizes cultures of modified embryonic stem cells injected into host embryos. The embryonic stem cells integrate into the host embryo and produce chimeras. Multiple matings are required to obtain transgenic animals. However, when a modified spermatid is injected into a mature egg, the resultant embryo will be completely heterozygous.

**[0035]** Accordingly, the invention provides a method for the creation of transgenic animals comprising obtaining a cell line according to the invention, generating a genetic modification by introduction of an exogenous nucleotide sequence, optionally selecting for stable incorporation of the transgene in the

chromosomal DNA, inducing spermatogenesis using SCF, using the resulting spermatids or sperm to fertilize an egg *in vivo* or *in vitro*. The transgene can be introduced using any appropriate technique recognized in the art. For example a transfection promoting agent, for example Lipofectamine® or the like, may be used or DNA may be introduced by transfection of naked DNA. In a preferred technique, the cultured cells of the cell line are exposed to naked DNA encoding the transgene or a genetic modification which can be incorporated by homologous recombination.

**[0036]** In an alternative embodiment of the method, spermatogonial cells of the invention that have been transfected with an exogenous nucleotide sequence can be transplanted into the testes of an animal sterilized by chemical or radiological sterilization. The recipient animal is then bred to produce transgenic offspring.

**[0037]** The transgenic animals produced by these methods will contain the transgene in cells throughout the animal. Thus, the transgeneic animals can be made to express the trangene or a targeted native gene in any, most, or all tissues according to the control elements included with the exogenous DNA, the insertion point, and/or the genetic modification provided by the exogenous DNA as will be recognized in the art. The foregoing methods can be likewise used for germ cell gene therapy, for example, to eliminate a heritable genetic disorder from the male line. Unlike the more common somatic gene cell therapies, these methods produce genetic alterations that are permanent and heritable.

**[0038]** The techniques and protocols referred to herein can be performed as practiced in the art except where modified herein or otherwise described in a reference. For routine practice of the protocols and techniques referenced herein, one of skill in the art is directed to the references cited in this application as well as the several *Current Protocols* guides, which are continuously updated, widely available and published by John Wiley and Sons, (New York). In the life sciences, Current Protocols publishes comprehensive manuals in Molecular Biology, Immunology, Human Genetics, Protein Science, Cytometry, Neuroscience,

Pharmacology, Cell Biology, Toxicology, and Nucleic Acid Chemistry. Additional sources are known to one of skill in the art.

**[0039]** A preferred mode of introducing either the DNA encoding the TERT subunit, any transgene, or both into the cells of the invention is through the use of a virus. "Virus", as used herein, means any virus, or transfecting fragment thereof, that can facilitate the delivery of the genetic material into cells. Examples of viruses that are suitable for use herein are adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, mumps virus, and transfecting fragments of any of these viruses, and other viral DNA segments that facilitate the uptake of the desired DNA segment by, and release into, the cytoplasm of cells and mixtures thereof. Other known vector systems, however, can also be utilized within the confines of the invention.

**[0040]** Exogenous nucleotide sequences, exogenous DNA sequences and "transgene", as used herein, means nucleotide sequences, not native to the recipient cell or animal, preferably DNA, capable of imparting novel genetic modification(s), or biologically functional characteristic(s) to the recipient. The novel genetic modification(s) or characteristic(s) can be encoded by one or more genes or gene segments, or can be caused by removal or mutation of one or more genes, and can additionally contain regulatory sequences. Examples of other mechanisms by which a gene's function can be expressed are genomic imprinting, i.e. inactivation of one of a pair of genes (alleles) during very early embryonic development, or inactivation of genetic material by mutation or deletion of gene sequences, or by expression of a dominant negative gene product, and the like, including expression of interfering RNA, expression of peptide aptamers and other means of modulating expression of a native gene or the activity of a native protein.

**[0041]** Overexpression of a gene is understood to mean production of substantial, or above average, quantities of the product encoded by the gene. Overexpression can refer to the expression of a native protein or polypeptide in a cell at levels greater than native levels, for example by activation of the native promoter or by introduction of exogenous nucleotide sequence encoding for the

protein driven by strong promoter. Overexpression can also refer to expression of substantial quantities of a non-native protein or polypeptide encoded on an exogenous nucleotide sequence driven by an inducible or constitutively active promoter.

**[0042]** In addition, genetic modification(s) can be artificially induced mutations or variations, or natural allelic mutations or variations of a gene(s). Mutations or variations can be induced artificially by a number of techniques, all of which are well known in the art, including chemical treatment, gamma irradiation treatment, ultraviolet radiation treatment, ultraviolet radiation, and the like. Chemicals useful for the induction of mutations or variations include carcinogens and others known in the art.

**[0043]** DNA segments of specific sequences can also be constructed to incorporate any desired mutation or variation or to disrupt a gene or to alter genomic DNA. Those skilled in the art will readily appreciate that the genetic material is inheritable and is, therefore, present in almost every cell of future generations of the progeny, including the germ cells.

**[0044]** Novel characteristics which can be provided by the method of the invention include, for example, the expression of a previously unexpressed trait, augmentation or reduction of an expressed trait, over expression or under expression of a trait, ectopic expression, that is expression of a trait in tissues where it normally would not be expressed, the attenuation or elimination of a previously expressed trait, combinations thereof, or the like. Other novel characteristics include, for example, the qualitative change of an expressed trait, for example, to palliate or alleviate, or otherwise prevent expression of an inheritable disorder with a multigenic basis.

**[0045]** The method of the invention is generally suitable for application to vertebrate animals, all of which are capable of producing sperm. Thus, in accordance with the invention, novel genetic modification(s) and/or characteristic(s) can be imparted to animals, including mammals, such as humans, non-human primates, for example, simians, domestic and agricultural animals such as sheep,

cows, pigs, horses, particularly race horses, dogs, cats, marine mammals, feral animals, rodents such as mice and rats, and the like. Other animals include fowl and the like.

**[0046]** A "transgenic" animal is one that has had exogenous DNA permanently introduced into its cells. The foreign gene(s) which (have) been introduced into the animal's cells is (are) also known as "transgene(s)". The present invention is applicable to the production of transgenic animals containing xenogeneic, i.e., exogenous, transgenic genetic material, or material from a different species, including biologically functional genetic material, in its native, undisturbed form in which it is present in the animal's germ cells. In other instances, the genetic material is "allogeneic" genetic material, obtained from different strains of the same species, for example, from animals having a "normal" form of a gene, or a desirable allele thereof. Also, the gene can be a hybrid construct consisting of promoter DNA sequences and DNA coding sequences linked together. These sequences can be obtained from different species or DNA sequences from the same species that are not normally juxtaposed. The DNA construct can also contain DNA sequences from prokaryotic organisms, such as bacteria, or viruses.

**[0047]** In a preferred embodiment, the transfected germ cells of the transgenic animal have the non-endogenous (exogenous) genetic material integrated into their chromosomes referred to as "stable transfection".

**[0048]** Those skilled in the art will readily appreciate that any desired traits generated as a result of changes to the genetic material of any transgenic animal produced by this invention are inheritable. Although the genetic material was originally inserted solely into the germ cells of a parent animal, it will be present in the germ cells of future progeny and subsequent generations thereof. The genetic material will also be present in the differentiated cells, i.e. somatic cells, of the progeny. This invention also encompasses progeny resulting from breeding of the present transgenic animals. The transgenic animals bred with other transgenic or non-transgenic animals of the same species will produce some transgenic progeny.

This invention, thus, provides animal line(s) which result from breeding of the transgenic animal(s) provided herein, as well as from breeding their fertile progeny.

**[0049]** "Breeding", in the context of this invention, means the union of male and female gametes so that fertilization occurs. Such a union can be brought about by natural mating, i.e. copulation, or by in vitro or in vivo artificial means. Artificial means include, but are not limited to, artificial insemination, in vitro fertilization, cloning and embryo transfer, intracytoplasmic spermatozoal microinjection, cloning and embryo splitting, combinations thereof, and the like. However, others can also be employed.

**[0050]** Following the methods described above, the skilled practitioner can make and use the invention without undue experimentation. The following examples are provided as illustrations of the methods and should not be construed as limiting the invention in any way.

## EXAMPLES

*Example 1: Making and characterizing an immortalized mouse spermatogonial cell line.*

**[0051]** We created cell lines using undifferentiated type A spermatogonia obtained from 6 day-old of Balb-c mice isolated by the STAPUT method (10). Since primary spermatogonia are difficult to transfect, we used a retrovirus system to introduce mTERT. The retrovirus construct was generated by inserting mTERT cDNA into the EcoR I site of the retrovirus vector pLXSN. A 3441 bp EcoR I/EcoR I fragment carrying 3395 bp of full-length murine telomerase reverse transcriptase (mTERT) cDNA was obtained from pGRN188 and subcloned into EcoR I site in a retrovirus vector pLXSN (Clontech Laboratories, Inc.). The orientation for expressing sense mTERT by 5' LTR was checked by digestion with Xho I. Expression of Neor was driven by SV40 early promoter. Viruses were harvested from supernatants of transfected Phoenix packaging cells. Isolated cells were infected with the retrovirus following a standard protocol (12). After two months of culture in the presence of G418, the cells expanded as they became

immortalized. Many of the cells possessed a morphology similar to primary type A spermatogonia, such as a large cell body, a spherical nucleus with a thin rim of cytoplasm and with perinuclear organelles (Fig. 1, A and B).

**[0052]** Immunocytochemistry showed that the cells also expressed c-kit, which is a biochemical marker for spermatogonia (Fig. 1C). Six clones, S1-S6, were obtained by subcloning from the immortalized mixed culture. After one year in culture, the cells (Fig. 1D) still displayed a similar morphology as freshly isolated type A spermatogonia and maintained the expression of telomerase as shown by Northern blot (Fig. 1E). To further confirm the germ cell characteristics of these cells, we checked for DAZL, which is a germ cell specific RNA-binding protein (13), and Oct-4, a germ cell specific transcriptional factor (14). As shown by RT-PCR and Western blot, all the cell lines were positive for both Oct-4 and DAZL (Fig. 1, F and G), respectively. Total RNA was isolated and cDNA synthesis carried out with random hexamers. RT-PCR amplification of Oct-4 using primers 5' agctgctgaagcagaagagg 3' and 5' ggttctcattgttgtcggct 3' (94°C for 35 sec,55°C for 30 sec, and 72°C for 45 sec; 30 cycles). The size of RT-PCR product was 207bp. Primers for RT-PCR amplification of protamine-2 are 5' gagcgcgtagaggactatgg 3' and 5' gcaagtgacttccttggctc 3' (94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec; 35 cycles). The product was 282 bp DNA fragment.

**[0053]** Oct-4 is expressed in totipotent embryonic cells. After gastrulation, Oct-4 expression becomes restricted to primordial germ cells (PGCs). In male mice, the expression of Oct-4 is maintained until the beginning of spermatogenesis and is confined to the type A spermatogonia (16) . Thus, these immortalized cells were likely derived from type A spermatogonia.

**[0054]** We further examined the physiological function of these cells by inducing differentiation with stem cell factor (SCF). In vivo, the type A spermatogonia go through mitoses forming many interconnected cells that enter meiosis (17). SCF and its receptor c-kit play a critical role in regulating this initial stage of spermatogenesis and trigger proliferation/differentiation of type A spermatogonia (18). A point mutation in c-kit, which impairs SCF-mediated

activation of phosphatidylinositol 3-kinase, leads to complete male sterility due to lack of differentiating spermatogonia (19, 20). All six cell lines demonstrated the presence of c-kit as shown by Western blot (Fig. 1H). We treated the S4 cells with mSCF to induce spermatogenesis in vitro. S4 cells responded to mSCF as shown by tyrosine phosphorylation of c-kit within 5 minutes of mSCF (100 ng/ml) treatment (Fig. 1I). We further analyzed the differential capacity of S4 cells to produce meiotic cells. The meiotic cell is distinguished from the mitotic cell by unique chromosome patterns, especially the assembly of the synaptonemal complex (SC) and the formation of chiasmata at the sites of crossover (genetic recombination). Immunofluorescence staining with an antibody against synaptonemal complex protein 3 (SCP3) demonstrated that mSCF induced the formation of SCs in the S4 cells within a week of culture (Fig. 2A). Moreover, crossovers also were found in the mSCF induced meiotic S4 cells shown by Giemsa staining (Fig. 2B). These SCF induced cells were also strongly positive for the meiosis-specific marker lactate dehydrogenase (LDH-C4) (21), (Fig. 2, C and D).

**[0055]** After two meiotic divisions, spermatocytes gave rise to haploid spermatids. We next used an Acr3-EGFP construct to test whether S4 cells differentiate into spermatids. Acr3-EGFP was made by inserting EGFP at the downstream site of a fused peptide of proacrosin signal fragment MVEMLPTVAVLVLAVSVVA and its N-terminal peptide KDNTT. The expression of the whole fusion protein is driven by the acrosin promoter. In Acr3-EGFP transgenic mouse lines, an accumulation of GFP was evident as tiny dots corresponding to proacrosomal granules at step 1 or 2 in spermatid differentiation. The green fluorescence merged to form one large granule and then flattened into a characteristic cap shape as spermiogenesis proceeded (22). The acrosin promoter is activated first in the cytoplasm of stage IV pachytene spermatocytes then continues throughout the early stages of spermiogenesis (23). Thus, Acr3-EGFP can be used to identify both meiotic and postmeiotic cells after introduction into our cell line. The stably transfected S4 cells were created by introducing Xba I linearized Acr3-EGFP-pcDNA3.1/Zeo(-).

**[0056]** Acr3-EGFP-pcDNA3.1/Zeo(-) was created by sub-cloning Xba I/Hind III fragment of Acr3-EGFP containing acrosin promoter, the MEMLPTVAVLVLAVSVVA- KDNTT-EGFP and bGH polyA from pUC19/Acr3-EGFP into pcDNA3.1/Zeo(-). After SCF stimulation, both meiotic cells and postmeiotic cells were observed by GFP (Fig. 2, E and F).

**[0057]** Some cells had a tiny green GFP dot at the cell pole similar to what was seen in postmeiotic cells in the Acr3-EGFP transgenic mice. These cells appeared to be step 1 or step 2 round spermatids. Some of these cells differentiated into more mature spermatids (Fig. 2, G to I) although sperm tails were not evident. The formation of an acrosome in SCF-induced cells was also revealed by differential interference contrast microscopy (Fig. 3A). We further examined the expression of the spermatid-specific markers SP-10 (25) and protamine-2 (26) in the SCF induced cell line. The cells expressed both SP-10 protein and protamine-2 analyzed by RT-PCR after two week of stimulation with mSCF (Fig. 3, B and C). Total RNA was isolated and cDNA synthesis carried out with random hexamers. RT-PCR amplification of Oct-4 using primers 5' agctgctgaagcagaagagg 3' and 5' ggttctcattgttgtcggct 3' (94°C for 35 sec,55°C for 30 sec, and 72°C for 45 sec; 30 cycles). The size of RT-PCR product was 207bp. Primers for RT-PCR amplification of protamine-2 are 5' gagcgcgtagaggactatgg 3' and 5' gcaagtgacttccttggctc 3' (94°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec; 35 cycles). The product was 282 bp DNA fragment.

**[0058]** Flow cytometry analysis was carried out to examine the number of chromosomes (ploidy) in the spermatogonial cell line. Spermatogonia contain the diploid (2N) number of chromosomes. As shown in Fig. 4B, the non-induced S4 cells were diploid and not aneuploid. Aneuploidy is the karyotype of transformed testicular cells (27). After one week of SCF induction, about 39% of the S4 cells were tetraploid (4N) characteristic of spermatocytes (Fig. 4C). After three weeks of induction, the number of haploid cells (1N) reached about 58% of the total cell population (Fig. 4D). Spermatids normally contain 1N number of chromosomes.

Thus, the S4 cells appear to differentiate into spermatocytes and round spermatids upon SCF stimulation. Figure 4A shows the ploidy from a normal testis.

**[0059]** Our mTERT immortalized mouse spermatogonial cells possess the properties of type A spermatogonial stem cells since they can proliferate to renew themselves and as well give rise to differentiated cells upon ligand stimulation. Bridges have not been noted between the dividing or the differentiating cells; the role of the bridges in vivo still remains to be elucidated.

*Example 2: In vitro fertilization using spermatids made from an immortalized mouse spermatogonial cell line.*

**[0060]** A spermatogonial cell line was obtained and cultured at 34° C in DMEM/F12 media as described above. The cells grow rapidly with a doubling time of about 72 hours. A portion of the cells was exposed to stem cell factor (100 ng/ml) added every 48 hours to the media for 2 to 3 weeks until about 33 % of the cells appeared as small round spermatids (about 7 micrometers).

**[0061]** Eggs were collected from the oviducts of super ovulated females following the procedures described by Kimura and Yanagimachi (32,33). The cells are freed from the surrounding cells by enzyme treatment and kept in CZB media for about and hour (34). The eggs activated by treatment with Ca-free CZB media containing 10mM $SrCl_2$. The activated eggs were rinsed and kept in CZB media for about 15 minutes at 37° C before injection of the round spermatids.

**[0062]** Differentiated cells were placed under oil. As shown in Fig. 5, the small round spermatids were selected and sequestered using a Piezo injection device for the injection into the eggs. As shown in FIG. 6, spermatids were injected into eggs using the procedure referred to as ROSI (round spermatid injection)(32). Between 5 and 6 hours after injection, the eggs were examined. Oocytes with two distinct pronuclei and a second polar body were retained and counted as normally fertilized. As shown in FIGS. 7 and 8, fertilized eggs were permitted to develop to stages which will be used for transfer to surrogate mothers.

*Example 3: Making an immortalized porcine spermatogonial cell line.*

**[0063]** The ability to manipulate the boar germ line is clinically important because of the potential for xenotransplantation to humans (35). Dobrinski and colleagues have shown that germ cell transplantation in the pig is feasible (1). Thus, there is the potential to create transgenic pigs using spermatogonial transplantation. Immortalized spermatogonial cell lines will also permit use of in vitro egg injection procedures.

**[0064]** We have previously reported the isolation of highly purified type A spermatogonial cells (36). Highly purified type A spermatogonial cells were isolated using a procedure essentially identical to the STAPUT technique (10). The purified cells were transfected with a vector encoding mouse TERT and cultured as described above. Cells were maintained in culture for more than 60 days. The cells maintained the appearance of spermatogonia seen in vivo in pigs. The experiment will be repeated using a vector encoding human TERT.

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WHAT IS CLAIMED IS:

1. A method for the creation of a spermatogonial cell line, the method comprising the steps of:

obtaining undifferentiated spermatogonial cells;

causing a catalytically active telomerase, or a catalytically active fragment thereof, to be overexpressed in the cells; and,

culturing the cells.

2. The method of claim 1 wherein the spermatogonial cells are purified.

3. The method of claim 1 or 2 wherein the overexpression of the catalytically active telomerase, or a catalytically active fragment thereof, is caused by introduction of an exogenous nucleotide sequence encoding the catalytically active telomerase or fragment.

4. The method of claim 1, 2 or 3, wherein the undifferentiated spermatogonial cells are type A spematogonial cells.

5. The method of any of claims 1-4, wherein the cells are obtained from an immature animal.

6. The method of any of claims 1-5, wherein the catalytically active telomerase, or catalytically active fragment thereof, comprises the telemorase reverse transcriptase (TERT) subunit.

7. The method of any of claims 1-6, wherein the overexpression of a catalytically active telomerase, or a catalytically active fragment thereof is caused by introduction of a nucleic acid comprising sequence encoding the telemorase

operably connected to a promoter capable of driving overexpression of the telemorase.

8. The method of any of claims 1-7, wherein the overexpression of a catalytically active telomerase, or a catalytically active fragment thereof is caused by introduction of a nucleic acid comprising sequence encoding the telemorase, or fragment thereof, and a selectable marker gene.

9. The method of clam 8, wherein the marker gene encodes an antibiotic resistance gene.

10. The method of claim 9, wherein the marker gene is a *neo* gene.

11. The method of any one of claims 8-10, further comprising selecting cells transfected by a nucleic acid comprising sequence encoding the telemorase, or fragment thereof, and a selectable marker gene.

12. A spermatogonial cell line created by the method of any one of claims 1-11.

13. A method for the creation of a mouse spermatogonial cell line, the method comprising the steps of:

obtaining undifferentiated type A spermtogonial cells from an immature male mouse by the STAPUT method;

introducing an exogenous nucleic acid comprising a sequence encoding the mTERT catalytic fragment of telomerase operably connected to a promoter sequence; and,

culturing the cells under conditions which permit the overexpression of the mTERT polypeptide.

14. The method of claim 13, wherein the cells are obtained at about 6 days post coitum.

15. The method of claim 13 or 14, wherein the exogenous nucleic acid is a vector.

16. The method of any of claims 13-15, wherein the exogenous nucleic acid is a retroviral vector.

17. The method of any of claims 13-16, wherein the exogenous nucleotide sequence further comprises a selectable marker gene.

18. The method of claim 17, wherein the selectable marker gene is a neo gene.

19. The method of claim 17 or 18, further comprising the step of selecting for transfected cells.

20. The method of claim 19, wherein selecting for transfected cells comprises exposing the cells to an antibiotic in culture.

21. The method of claim 19, wherein selecting for transfected cells comprises exposing the cells to G418 in culture.

22. A mouse spermatogonial cell line created by the method of any of claims 13-21.

23. A mouse spermatogonial cell line of claim 12 or 22 that has a morphology substantially the same as newly isolated spermatogonial cells after about two months or more of cell culture.

24. A mouse spermatogonial cell line of claim 23 that has a morphology substantially the same as newly isolated spermatogonial cells after a year or more of cell culture.

25. A method for forming spermatids comprising exposing spermatogonial cells obtained according to any of claims 1-11, 13-21, or cells of the spermatogonial cell lines of any of claims 12, 22, 23, or 24 to stem cell factor.

26. The method of claim 25, wherein the stem cell factor is the native stem cell factor of the species from which the cells were obtained.

27. The method of claim 25 or 26, wherein the cells are exposed to stem cell factor at a concentration of about 100 ng/ml.

28. The method of claim 25, 26 or 27, wherein about 50% or more of the cells have formed haploid spermatids about 3 weeks after initiation of contacting the cells with stem cell factor.

29. A method of investigating the effect of a genetic modification on spermatogenesis comprising:

obtaining spermatogonial cells according to any of claims 1-11, 13-21, or cells of the spermatogonial cell lines of any of claims 12, 22, 23, or 24;

genetically modifying the cells or the expression of a gene of the cells;

exposing the cells to stem cell factor; and

observing the cells.

30. A method of investigating the effect of a compound of interest on spermatogenesis comprising:

obtaining spermatogonial cells according to any of claims 1-11, 13-21, or cells of the spermatogonial cell lines of any of claims 12, 22, 23, or 24;

exposing the cells to the compound;

exposing the cells to stem cell factor; and

observing the cells.

31. A method for the creation of transgenic animals comprising:

obtaining spermatogonial cells according to any of claims 1-11, 13-21, or cells of the spermatogonial cell lines of any of claims 12, 22, 23, or 24;

transfecting the cells with an exogenous nucleotide sequence;

inducing spermatogenesis in the cells by exposing the cells to stem cell factor to form spermatids; and,

causing an egg to be fertilized by the spermatids.

32. A transgenic animal produced by the method of claim 31.

33. The progeny of the transgenic animals of claim 32 that have inherited a genetic modification caused by the exogenous nucleotide sequence.

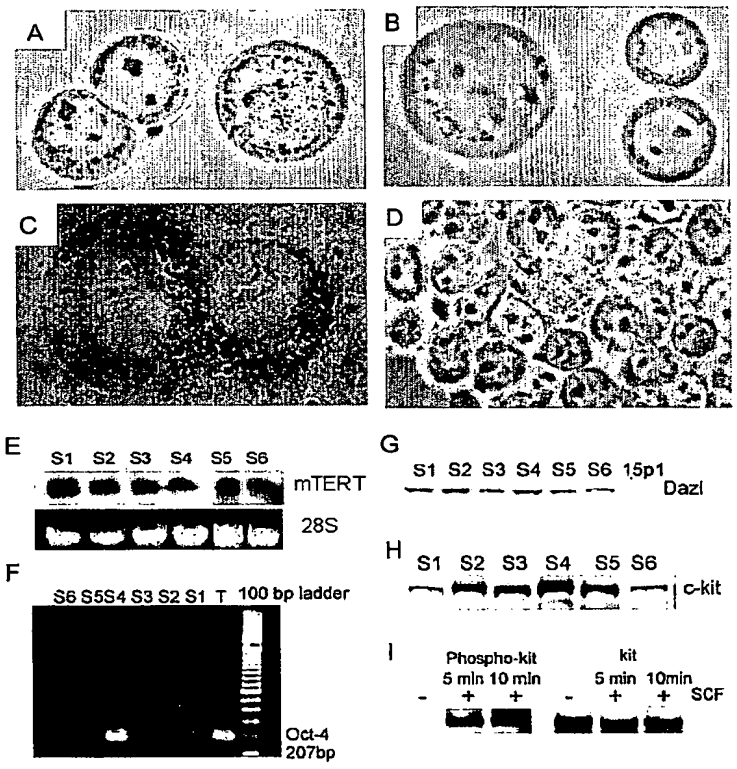

A
B
C
D
E
S1 S2 S3 S4 S5 S6
mTERT
28S
F
S6 S5S4 S3 S2 S1 T 100 bp ladder
Oct-4
207bp
G
S1 S2 S3 S4 S5 S6 15p1
Dazl
H
S1 S2 S3 S4 S5 S6
c-kit
I
Phospho-kit
5 min 10 min
kit
5 min 10min
- + + - + + SCF

FIG. 1

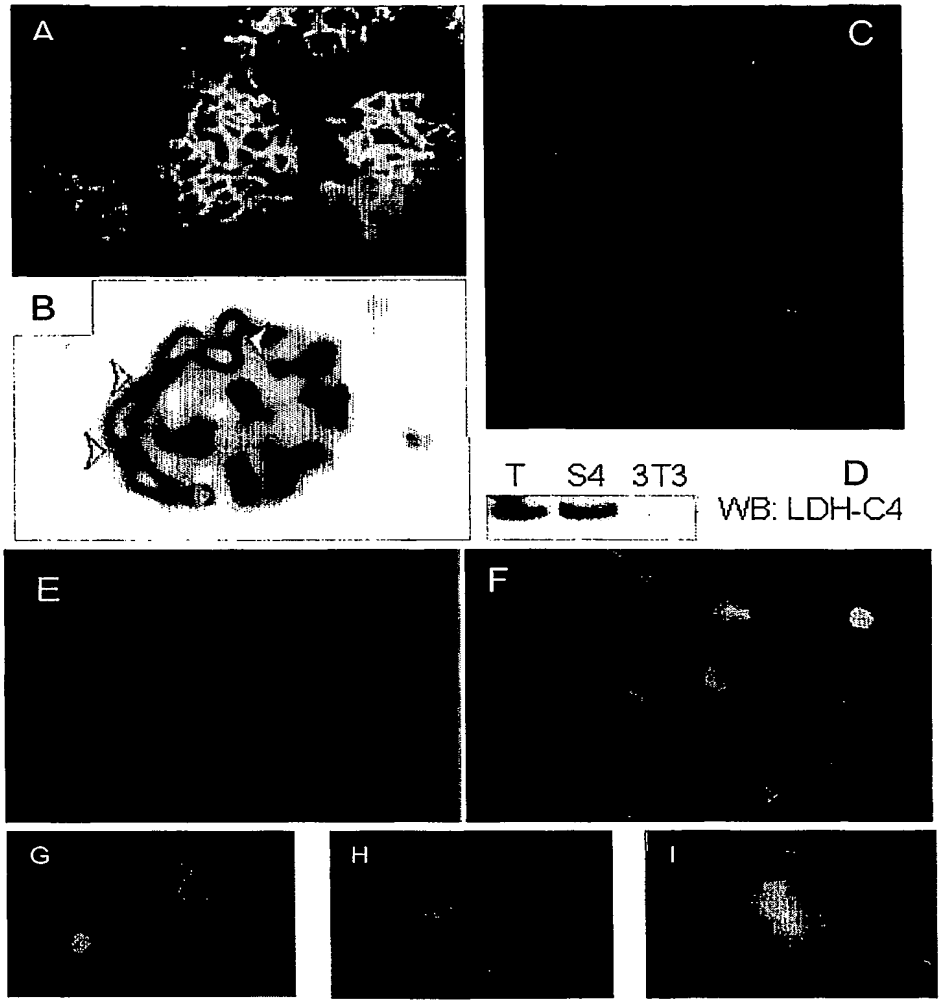



FIG. 2

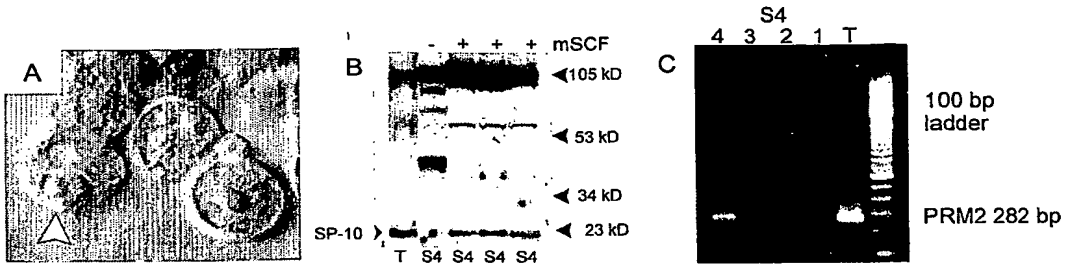

A
B
- + + + mSCF
105 kD
53 kD
34 kD
SP-10
23 kD
T S4 S4 S4 S4
C
S4
4 3 2 1 T
100 bp ladder
PRM2 282 bp

FIG. 3

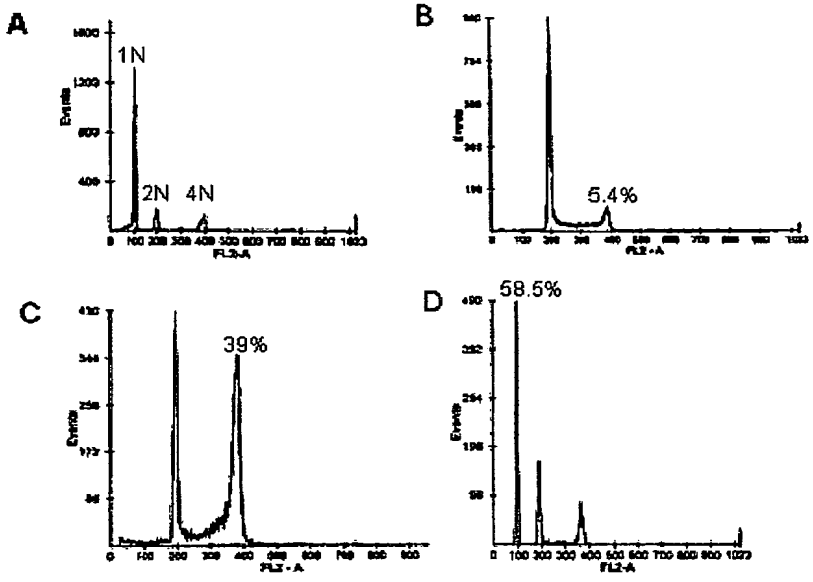



FIG. 4

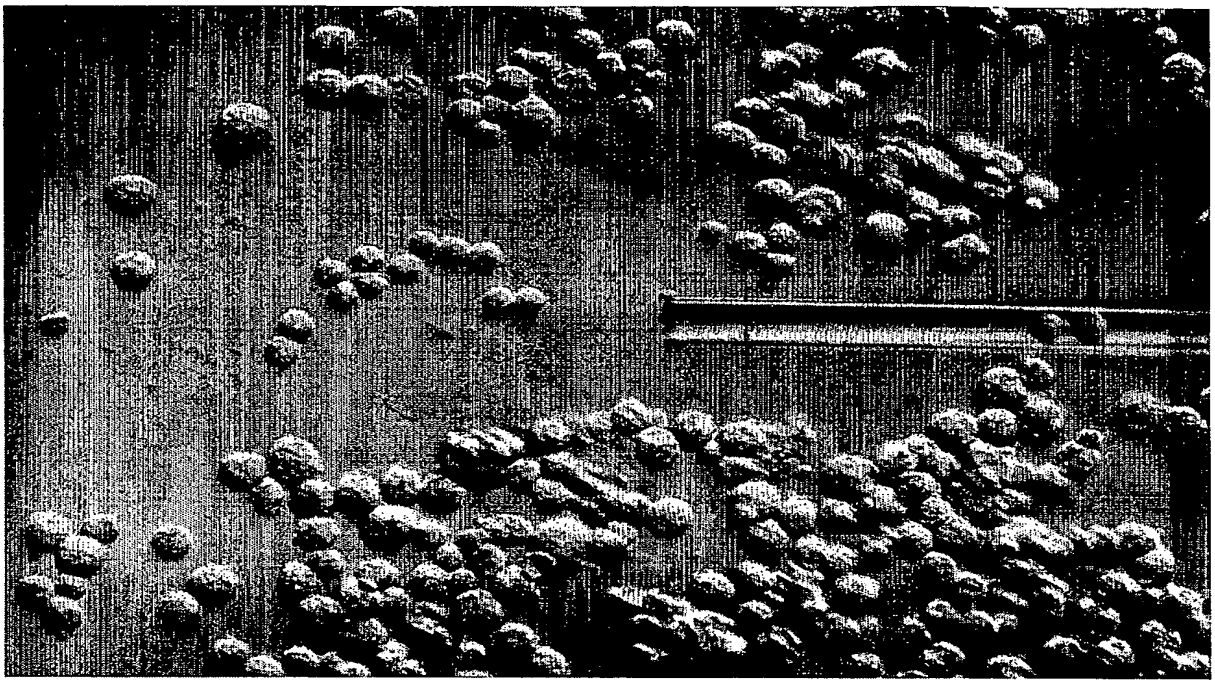
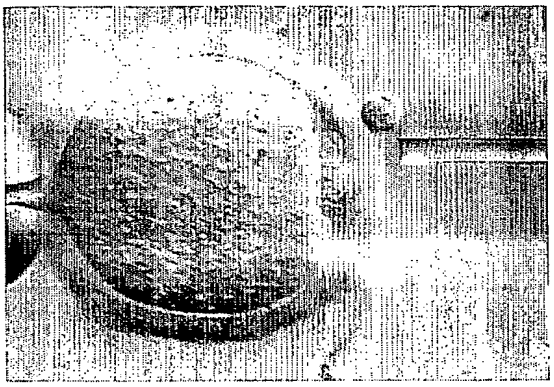
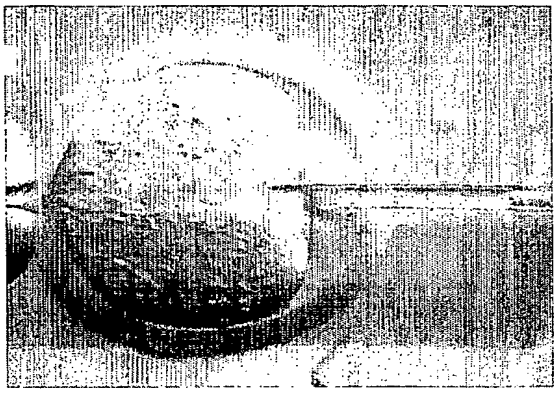


FIG. 5



A



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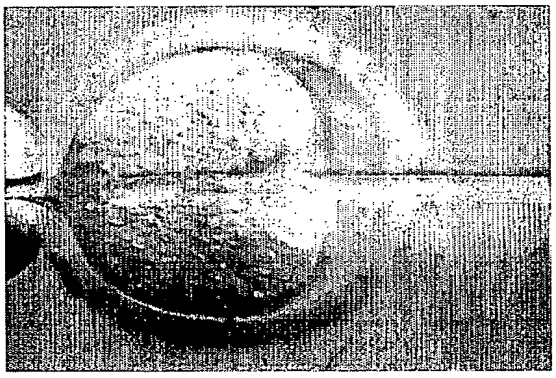
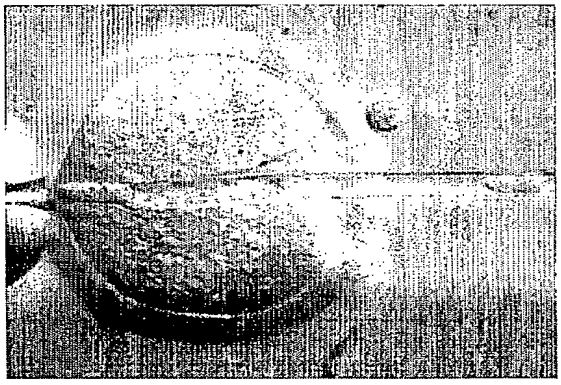


C



D

Fig. 6

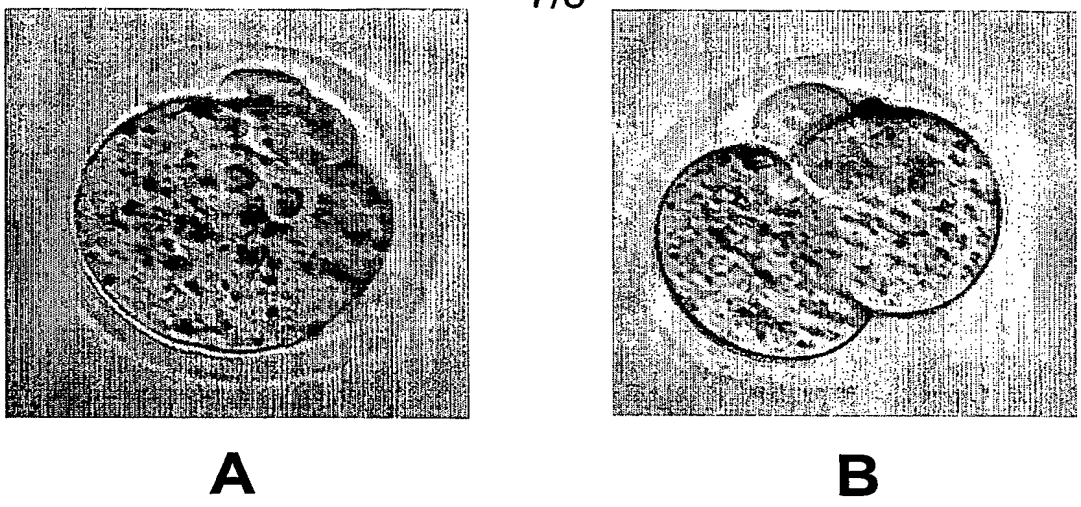



Fig. 7

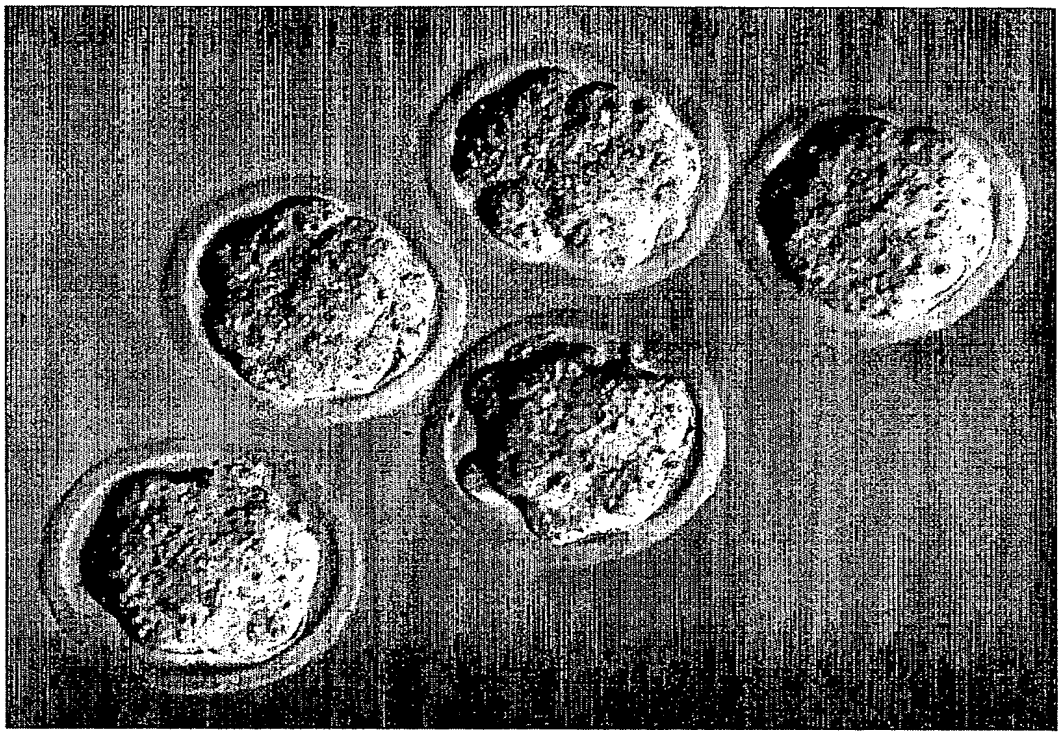


FIG. 8